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14. ABSTRACT

The goal of my DOD-supported research is determine the role of the new mTOR complex (mTORC2) in Autism Spectrum Disorder (ASD). ASD individuals exhibit impaired social interactions, seizures and abnormal repetitive behavior. In addition, 70-80% of autistic individuals suffer from mental retardation. Autism is a heritable genetically heterogeneous disorder and mutations in negative regulators of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, such as PTEN were associated with ASD. Here, we show that in the hippocampus of Pten fb-KO mice – where Pten is conditionally deleted in the murine forebrain – the activity of both mTORC1 and mTORC2 is increased. In addition, Pten fb-KO mice exhibit seizures, learning and memory and social deficits. Our remarkable preliminary data show that genetic inhibition of mTORC2 activity in Pten-deficient mice significantly promotes survival. In addition, Pten-rictor fb- double KO (DKO) mice, in which mTORC2 activity is restored to normal levels, EEG seizures, learning and memory as well as social phenotypes, are all rescued. In the second year, we will study the molecular mechanism underlying this process. These insights hold the promise for new treatment of ASD.

15. SUBJECT TERMS Autism Spectrum Disorder (ASD), mTORC2, mTORC1, protein synthesis, actin polymerization, mitochondria function, long-term memory, social behaviors, repetitive behaviors, and seizures.

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1. Introduction:

Autism represents a heterogeneous group of disorders, which are defined as "autism spectrum disorders" (ASDs). ASD individuals exhibit common features such as impaired social interactions, language and communication, and abnormal repetitive behavior. In addition, 70-80% of autistic individuals suffer from mental retardation[1-3]. The major goal of this award is to determine the role of mTORC2 in two mouse models of ASD. Recently, we have shown that mTORC2 plays a crucial role in long-term memory formation[4]. Briefly, mice lacking mTORC2 showed impaired long-lasting changes in synaptic strength (L-LTP) as well as impaired long-term memory (LTM). In addition, we have found that by promoting mTORC2 activity, with a new agent A-443654, it facilitates L-LTP and enhances long-term memory formation in WT mice. Interestingly, mTORC2 activity is altered in both ASD patients and ASD mouse models harboring mutation in *Tsc* and *Pten*[5,6]. Hence, in this proposal we will test the hypothesis that the neurological dysfunction in several ASD mouse models is caused by dysregulation of mTORC2 rather than mTORC1 activity.

2. Keywords: Autism Spectrum Disorder (ASD), mTORC2, mTORC1, protein synthesis, actin polymerization, mitochondria function, long-term memory, social behaviors, repetitive behaviors, seizures.

3. Overall Project Summary. We have generated a remarkable amount of progress regarding specific Aim 2. Given that mTORC2 activity is increased in Pten-deficient mice[7,8], we wondered whether its reduction could rescue the ASD-like cellular and behavioral phenotypes in PTEN-deficient mice. We cannot block mTORC2 activity pharmacologically because specific inhibitors of mTORC2 are not available. Hence, we conditionally deleted Pten and/or rictor in the murine forebrain using the Cre/lox system. Because the αCaMKII promoter is inactive before birth[9,10], this manipulation rules out possible developmental defects caused by the lack of of pten and *rictor*. We thus generated and studied the followings experimental mice: WT mice, *Pten* forebrain-specific knockout (here defined as *Pten* fb-KO), *Pten-rictor* forebrain-specific double knockout (here defined as *Pten-Rictor* fb-DKO). According to our preliminary data, mTORC2-mediated phosphorylation of Akt at Ser473 [an established readout of mTORC2 activity[6] and mTORC1-mediated phosphorylation of ribosome protein S6 [a well-established readout of mTORC1 activity [11]] were both increase in the hippocampus of *Pten* fb-KO (**Fig. 1)**. By contrast, in *Pten-rictor* fb-DKO only mTORC1 activity is up-regulated (**Fig. 1**). Hence, conditional deletion of *rictor* selectively blocks mTORC2 activity in Pten-deficient neurons.

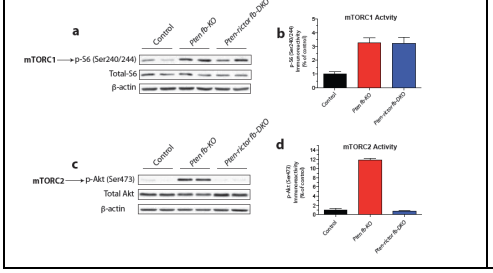

Fig. 1. mTORC2 activity is selectively suppressed in the hippocampus from *Pten-rictor* fb-KO mice. a, b. Western blots reveals that mTORC1 activity (p-S6 Ser240/244) is unaltered in the brain of *Pten-rictor* fb-KO mice (n=5) compared to *Pten* fb-KO mice (n=5). **c,d.** A significant decrease in mTORC2 activity (p-Akt-Ser-473) is observed in the hippocampus of *Pten-rictor* fb-KO mice, compared to *Pten-rictor* fb-KO mice.

Kaplan-Meier analysis of animal survival revealed a dramatic decreased in survival in *Pten* fb-KO mice (**Fig. 2;** *Pten* fb-KO mice die at an age of 52,4 +/- 8 days), which was remarkably prolonged by genetically inhibiting mTORC2 (**Fig. 2;** *Pten-Rictor* fb-DKO die at an age of 119.4 +/- 25). Hence, inhibition of mTORC2 in Pten-deficient mice prolongs their survival.

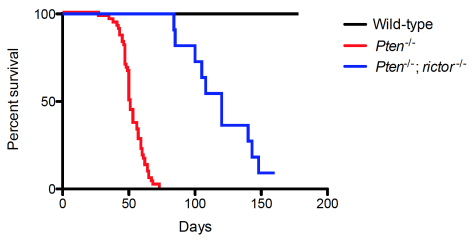

Fig. 2. Genetic inhibition of mTORC2 prolongs survival in *Pten*-deficient mice. Kaplan-Meier survival curves for wild-type, Pten fb-KO and Pten-rictor fb-KO. The median survival for *Pten-rictor* fb-DKO (n=35) is 120 days, a 140% extension in life span compared with the single Pten- fb-DKO mice (n=11). The curves of *Pten* fb-KO mice and *Pten-rictor* DKO mice are significantly different ($p<0.0001$).

Because patients with Pten mutations exhibit seizures, we next analyzed spontaneous seizures and abnormal electroencephalogram (EEG) activity. We found that *Pten* fb-KO mice show abnormal EEG as well as tonic-clonic seizures (**Fig. 3**). By contrast, while *Pten-Rictor* fb-DKO showed some abnormalities (spikes) in the EEG pattern, they did not show tonic-clonic and/or EEG seizures (**Fig. 3**). Thus, inhibition of mTORC2 suppresses the seizures EEG phenotype in Pten-deficient mice.

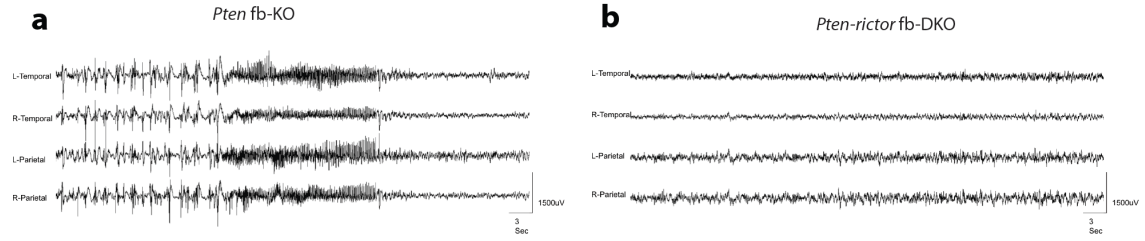

Fig. 3. Genetic inhibition of mTORC2 suppresses EEG seizures *Pten* fb-KO mice. Traces from bilateral cortical electrodes (Left hemisphere, L, Right hemisphere, R) show abnormal EEG seizures in freely moving *Pten* fb-KO mice (**a;** n=2), which are suppressed in *Pten-rictor* fb-DKO mice (**b;** n=2).

Finally, we measured learning and memory and social phenotypes in *Pten* fb-KO mice and *Pten-Rictor* fb-DKO. As expected, *Pten* fb-KO mice showed impaired long-term fear memory (**Fig. 4a**) and deficient social interaction (**Fig. 4b**). However, both long-term memory and social deficits are rescued in *Pten-rictor* fb-DKO (**Fig. 4a-b**). We are currently measuring whether direct social interaction and repetitive behaviors are also rescued by inhibition of mTORC2 in *Pten*-deficient mice.

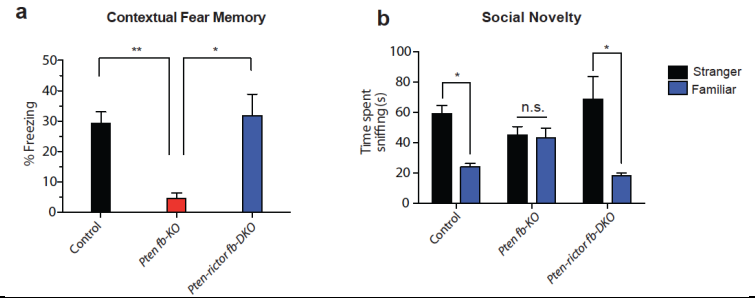

Fig. 4. Genetic inhibition of mTORC2 rescues the deficient long-term memory and social novelty in Pten fb-KO mice. a, For contextual fear conditioning, freezing times were recorded 24 hr after conditioning. As compared to control mice (n=29), Pten fb-KO mice show deficient freezing 24 hr after training, indicating that their long-term memory is impaired. In Pten-rictor fb-DKO mice, freezing levels are similar to those observed in WT mice, indicating that their long-term memory is restored. **b,** Social behavior test: time spent interacting with either a stranger or familiar mouse. WT mice spent most of the time interacting with the stranger mouse but *Pten* fb-KO mice spent equal time interacting with the familiar or strange mouse, indicating that social behavior is impaired in these mice. Like WT mice, *Pten-rictor* fb-KO mice spent more time interacting with the stranger mouse, indicating that social behavior is restored.

The goal of subaim-2 was to increase mTORC2 activity by treating Tsc2-deficient mice with the mTORC2 agonist A-443654[4]. We have obtained Tsc2 floxed mice but after breeding we noticed that they were in a mix background. Hence, to avoid any behavioral artifacts due to mixed genetic background, we decided to backcross these mice to C57BL6 mice to obtain a pure backgound. It is noteworthy that genetic background may alter behavior of autistic mice[12]. We have now obtained mice in a pure background and these mice are now being crossed with CamKII-Cre. In the next few months or so, Tsc2-deficient mice will be treated with vehicle and A-443654 and behavior and electrophysiology will be performed in these mice, as discussed in the proposal.

4. Key Research Accomplishment

- We developed a way to specifically block mTORC2 activity in Pten-deficient mice.
- Genetic deletion of mTORC2 prolongs the survival of Pten-deficient mice.
- Genetic deletion of mTORC2 dramatically attenuates seizures in Pten-deficient mice.
- Genetic deletion of mTORC2 improves cognitive and social phenotypes in Pten-deficient mice.

5. Conclusion

It has been proposed that the increased mTORC1 in Pten-deficient or Tsc-deficient mice causes the cellular and behavioral phenotypes associated with ASD[13-19]. Our new data challenge this view and posit that the neurological dysfunction in ASD, at least in the Pten-ASD mouse model, is caused by dysregulation of mTORC2. Hence, these preliminary data are very important since they identified a new signaling pathway involved in ASD and seizure disorders that could be targeted and lead to the development of new treatments for ASD and seizure disorders.

Future experiments: We we will study the precise mechanism by which mTORC2 suppresses Pten-mediated cellular and behavioral phenotypes. We will emphasize in two major mechanisms. First, given that mTORC2 regulates actin polymerization, we will measure changes in actin polymerization in *Pten* fb-KO and *Pten-rictor* fb-DKO mice. Second, given that Pten and mTORC2 both are localized in mitochondria, we will determine whether Pten-deficient brains exhibit impaired mitochondria respiration and such an impairment is rescued in *Pten-rictor* fb-DKO brain.

In addition, we will measure whether inhibitory/excitatory balance in disrupted in *Pten* fb-KO and rescued in *Pten-rictor* fb-DKO mice.

Finally, we will start to characterize the vehicle-treated and A-443654-treated Tsc-deficient mice in behavior and electrophysiology.

6. Publications
Some of these data described above were presented in a recent symposium: "Catastrophic Epilepsy" at the Neurological Research Institute (NRI), Houston, Texas

7. Inventions, Patents and Licenses.
Nothing to report

8. Reportable Outcome
Nothing to report

9. Other achievements
We developed forebrain-specific rictor-Pten double KO mice.

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11. Appendices

N/A